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Behavioural mechanisms of reproductive isolation between two hybridizing dung fly species

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Characterization of the phenotypic differentiation and genetic basis of traits that can contribute to reproductive isolation is an important avenue to understand the mechanisms of speciation. We quantified the degree of prezygotic isolation and geographical variation in mating behaviour among four populations of *Sepsis neocynipsea* that occur in allopatry, parapatry or sympatry with four populations of its sister species *Sepsis cynipsea*. To obtain insights into the quantitative genetic basis and the role of selection against hybrid phenotypes we also investigated mating behaviour of F_1 hybrid offspring and corresponding backcrosses with the parental populations. Our study documents successful hybridization under laboratory conditions, with low copulation frequencies in heterospecific pairings but higher frequencies in pairings of F_1 hybrids signifying hybrid vigour. Analyses of F_1 offspring and their parental backcrosses provided little evidence for sexual selection against hybrids. Longer copulation latencies in heterospecific pairings indicate species recognition, probably due to surface or volatile chemicals. The frequency of male mating attempts did not differ greatly between species or hybrid pairings, suggesting no male discrimination of mating partners. Female shaking duration, signifying female choice and/or reluctance to mate, differed strongly between the species and appears to contribute to avoiding heterospecific males; this trait is partially maternal inherited. Importantly, females of both species discriminated more strongly against males in areas of sympatry than allopatry indicating reinforcement. Shorter copulations in heterospecific parental pairings and longer copulations in F_1 hybrids suggest mechanistic difficulties with sperm transfer. Overall, our study highlights an important role of character displacement affecting mating behaviour of hybridizing sepsid species in geographical areas of coexistence.

Speciation proceeds gradually from restricted levels of gene flow at early stages to complete reproductive isolation at later stages (Coyne & Orr, 2004; Dobzhansky, 1951; Mayr, 1942). In many cases ecological, spatial or temporal niche differentiation prevents interbreeding between hybridizing species (Schluter, 2000, 2001). More interestingly, reproductive isolation may evolve through sexual selection leading to divergence in mate or gamete recognition systems (Kozak, Reisland, & Boughmann, 2009; Svensson, Karlsson, Friber, & Eroukhmanoff, 2007; Via, 2001). While theoretical studies have established sexual selection as an important potential agent in driving the evolution of reproductive isolation (Gavrilets, 2000; Lande, 1981; Turelli, Barton, & Coyne, 2001), supporting empirical data remain scarce and largely restricted to phylogenetic species comparisons over long evolutionary timescales (Kraaijeveld, Kraaijeveld-Smit, & Maan, 2010; Panhuis, Butlin, Zuk, & Tregenza, 2001). As a consequence, for many taxa it is unclear whether sexual selection alone causes reproductive isolation independent of species composition within habitats, or whether it acts in a more punctuated manner as predicted for reproductive character displacement in geographical areas of coexistence (Gavrilets, 2000; Lande, 1981; Turelli et al., 2001). In this context, several authors have recently emphasized the need to better understand the relationship between micro-evolutionary mechanisms causing trait divergence and macro-evolutionary patterns among lineages showing some degree of reproductive isolation.

Behavioural, morphological (i.e. mechanical) or olfactory differences between incipient species can lead to strong prezygotic isolating barriers, which, however, may remain incomplete. The main, and therefore the strongest, barriers result from postzygotic isolation with reinforcement, fertilization problems and hybrid male sterility (Hood, Egan, & Feder, 2012; Reed & Markow, 2004;

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Wassermann & Koepfer, 1977). Although reproductive isolation involves many different types of traits, behaviour is considered to be one of the main driving forces behind the evolution of reproductive barriers to gene flow (Gleason & Ritchie, 1998; Puniamoorthy, Ismail, Tan, & Meier, 2009; Shaw & Herlihy, 2000). For example, Puniamoorthy (2014) demonstrated for the neotropical fly *Archisepsis diversiformis* that qualitatively different courtship behaviours contributed to reproductive isolation between two geographically separated populations otherwise presenting only minor morphological and molecular differentiation.

The closely related sister species *Sepsis cynipsea* and *Sepsis neocynipsea* (Diptera: Sepsidae) offer great opportunity to investigate behavioural mechanisms and underlying evolutionary forces leading to reproductive isolation at early stages of speciation (Via, 2009). Based on their partially sympatric distribution in the Swiss Alps and strong similarities in morphology and behaviour we suspected that these two species might hybridize in nature. In this study, we examined typical mating traits in conspecific versus heterospecific parental pairings, F_1 hybrids and backcrosses between Swiss sympatric, European parapatric and North American allopatric populations, focusing on behavioural traits common to both species: male mating attempts by jumping on a partner; female shaking during pairing, here probably indicating male assessment; and copulation frequency, latency and duration (Blanckenhorn, Mühlhäuser, Morf, Reusch, & Reuter, 2000; Parker, 1972a, 1972b; Ward, 1983). Although the reluctance and assessment functions of female shaking can be hard to distinguish in practice (Blanckenhorn et al., 2000), we expected more pronounced female mate choice in heterospecific pairings following male assessment and species recognition, eventually resulting in reluctance to mate. We further expected the lowest hybridization rates and strongest (i.e. reinforced) behavioural differentiation in the European sympatric populations of the Swiss Alps, and some differentiation between European and North American *S. neocynipsea* due to their spatial separation.

METHODS

Study Organisms

Sepsis cynipsea and *S. neocynipsea* are two closely related species that exhibit clear morphological and behavioural differences (Pont & Meier, 2002) but limited variation in gene sequence data indicating differentiation (Baur, Schäfer, Blanckenhorn, & Giesen, 2017; Puniamoorthy, Su, & Meier, 2008; Su, Kutty, & Meier, 2008). *Sepsis cynipsea* is the most common sepsid species in north-central Europe, while populations of *S. neocynipsea* are present in Europe only in the Alps and other mountainous regions, whereas in North America they abound also at low altitudes, there occupying the ecological niche of the absent *S. cynipsea* (Pont & Meier, 2002). Both similarly breed in fresh cowpats and are reproductively active from spring to late autumn (Eberhard, 1999; Parker, 1972a, 1972b; Pont & Meier, 2002). While the mating system of *S. cynipsea* is well studied (Blanckenhorn, Morf, Mühlhäuser, & Reusch, 1999; Ding & Blanckenhorn, 2002; Hosken, Martin, Born, & Huber, 2003; Parker, 1972a, 1972b; Puniamoorthy et al., 2009; Rohner, Blanckenhorn, & Puniamoorthy, 2016; Ward, 1983; Ward, Hemi, & Rösli, 1992), little is known about its sister species *S. neocynipsea* (Eberhard, 1999; Puniamoorthy et al., 2009; Rohner et al., 2016).

Ethical Note and Maintenance of Flies

No legal regulations for scientific laboratory work with sepsid flies exist in Switzerland, the EU or the U.S.A. and no licences or permits were needed. We caught wild individuals by swiping a butterfly net over fresh cowpats. Sepsid flies were extracted from the net using an aspirator and transferred into 1-litre transparent plastic containers with fixed Eppendorf tubes offering sugar and water ad libitum. Most other nontarget insects so collected were released again on site. Collected live flies were brought or sent to our laboratory, where they were identified by sex and species according to differences in male armoured foreleg morphology. Male flies were stored as voucher specimens in 100% ethanol at −20 °C, and gravid females were isolated into round 50 ml glass vials including a rectangular plastic dish ($4.2 \times 2.1 \times 1.6$ cm^3) filled with fresh cow dung as oviposition substrate and some grains of sugar. Emerging F_1 offspring of single females were then transferred into $1 \times 1 \times 1.4$ dm^3 plastic containers with fresh cow dung, water ad libitum and sugar for continuous propagation in the laboratory. Isofemale lines were subsequently held in these containers in a climate chamber at 24 °C, 60% humidity and 16:8 h light:dark cycle; fresh cow dung was provided every 14 days (rearing conditions are detailed in Puniamoorthy, Schäfer, & Blanckenhorn, 2012). We identified species in isofemale lines according to their male F_1 offspring. Our experimental flies were derived from isofemale lines that had been housed and propagated for up to 2 years before our experiment (see Rohner et al., 2016, for more details). After experiments we froze all flies in 100% ethanol at −20 °C.

Fly Origin and Pairing Scheme

Wild-caught gravid females were collected from six sites (i.e. populations) to ultimately establish 5–15 isofemale lines per population in the laboratory (Table 1). *Sepsis cynipsea* and *S. neocynipsea* were obtained from two areas in Switzerland where the two species are sympatric (Zurich, Sörenberg). *Sepsis cynipsea* were further collected from another two European sites, where we did not observe *S. neocynipsea* (Ludwigshafen, Germany, and Stirling, U.K.). However, there are records of *S. neocynipsea* near these sites (Ozerov, 2005; Pont & Meier, 2002), so we classified these populations as parapatric. The other *S. neocynipsea* originated from two allopatric North American populations where *S. cynipsea* does not exist (Fort Hall, ID, and Lamar Valley, WY).

With these flies, we could thus form reciprocal heterospecific parental pairings of three biogeographical types with two population replicates each: European sympatry, European parapatry and cross-continental allopatry (Table 2). In parallel, we performed conspecific parental pairings within each of the four populations per species as the baseline for comparison, as well as two reciprocal population replicates of European with North American *S. neocynipsea* as conspecific allopatric cross-continental pairings (Table 2). In all cases, one population replicate consisted of 15–20 pairing replicates derived from our isofemale lines. Potentially

Table 1
Biogeographical origin of isofemale lines per population of the study species

Biogeographical origins (code)		
S. cynipsea	*S. neocynipsea*	Coordinates
Switzerland, Zurich (CH_1)	Switzerland, Zurich (CH_1)	47°24′0.60″N, 8°34′23.97″E
Switzerland, Sörenberg (CH_2)	Switzerland, Sörenberg (CH_2)	46°49′23.72″N, 8°1′54.59″E
U.K., Stirling (EU_1)		56°6′59.47″N, −3°56′12.83″W
Germany, Ludwigshafen (EU_2)		49°28′41.25″N, 8°22′21.65″E
	Idaho, Fort Hall (NA_1)	43°1′59.69″N, −112°26′17.91″W
	Wyoming, Lamar Valley (NA_2)	44°52′6.67″N, −110°10′28.72″W

EU = Europe; CH = Switzerland; NA = North America.

Table 2
Pairing scheme of three biogeographical types (female × male)

Biogeographical type	Pairings	Population replicates
Sympatry in Europe	EU *S. neocynipsea*×EU *S. cynipsea*	$(CH_1)\times(CH_1)$ $(CH_2)\times(CH_2)$
Parapatry across Europe	EU *S. neocynipsea*×EU *S. cynipsea*	$(EU_1)\times(CH_1)$ $(EU_2)\times(CH_2)$
Allopatry across continents		
Interspecific	NA *S. neocynipsea*×EU *S. cynipsea*	$(NA_1)\times(CH_1)$ $(NA_2)\times(CH_2)$
Intraspecific	NA×EU *S. neocynipsea*	$(NA_1)\times(CH_1)$ $(NA_2)\times(CH_2)$

Interspecific (three groups) and intraspecific (one group) pairing scheme. All pairings were reciprocal. Population codes as in Table 1.

lower sample sizes in mating experiments with F_1 hybrid offspring were expected due to difficulties in obtaining hybrids. For backcrosses, we aimed for a sample size of six replicates per pairing, as we set up two reciprocal types (female hybrid with male parental: $F_1 \times P$; female parental with male hybrid: $P \times F_1$) to detect possible sex-specific effects. In the end, we conducted observations for (1) conspecific and heterospecific parental (P) pairings (mean sample size = 19.13, range 15–20), (2) F_1 hybrid (F_1) pairings using the offspring resulting from heterospecific pairings (mean sample size = 11.44, range 3–20) and (3) backcrosses (BC) of F_1 hybrid offspring with the parental species (mean sample size = 4.23, range 1–6). All pairings were done reciprocally.

Hybrid flies for our behavioural assessments of the F_1 and backcrosses were generated by randomly combining up to 30 flies of one sex from various isofemale lines of a given population and species with a roughly equal number of flies of the other sex from various isofemale lines of a given population of the other species (Table 2; done reciprocally). Matings in this setting were necessarily heterospecific, and females were allowed to oviposit eggs into fresh cow dung to generate F_1 hybrid offspring for our experiments.

Assessment of Mating Behaviour

For each pairing replicate (see above) we combined five virgin females with five virgin male individuals (i.e. 5f:5m) into a round 50 ml (length 8 cm × diameter 2.5 cm) glass vial containing a smear of cow dung, all independently and randomly chosen from the various isofemale lines of a given population. This implies that some of the individuals in each replicate vial may have stemmed from the same isofemale line by chance. Virginity was guaranteed by separating flies by sex within 24 h after emergence. Flies were always aged 3–6 days after adult eclosion to ensure sexual maturity (Teuschl & Blanckenhorn, 2007). Owing to errors, losses, deaths or accidental surplus of individuals, effective group sizes varied between 3f:3m and 6f:6m. We thus followed Puniamoorthy (2014), who reported for *A. diversiformis* that hybrids between different sepsid species were produced only when flies were in groups, thus emulating the natural situation at cowpats where the probability of interaction was high (Eberhard, 1999; Parker, 1972a, 1972b).

Observation of mating behaviour started right after fly introduction and lasted for 30 min. We recorded (1) the total number of male mating attempts as an indicator of male willingness to mate, i.e. jumps onto a female, (2) the cumulative female shaking duration (s) with a mounted male indicating mate assessment and/or reluctance to mate, and (3) the average duration (min) of all copulations (Blanckenhorn et al., 2000; Boake, Price, & Andreadis, 1998; Ding & Blanckenhorn, 2002). We always scored the entire copulation duration, even if it exceeded the 30 min observation interval. From these assays, we further derived for final analysis (4) the time to first copulation (i.e. copulation latency) as an indicator of how fast mating ensues and (5) the number of copulations realized per male mating attempt (copulation frequency). These data can be extracted from the Supplementary Material.

Statistical Analyses

We ultimately standardized all trait measurements (except copulation duration) for analysis to 30 min and one pair. The number of male mating attempts, female shaking duration, copulation duration and latency were $\log_{10}$-transformed for a better residual distribution in parametric statistical tests. Mating frequency was arcsine-transformed for analysis (logistic analyses with binomial errors yielded qualitatively similar results). All five traits were analysed separately, with and without the other traits as covariates because male and female behaviours interact to produce matings (only significant covariates are reported in the Results), with univariate general linear models (GLMs) in SPSS Statistics Version 23 (IBM, Armonk, NY, U.S.A.). For the parental pairings, a given behavioural trait was analysed as a function of species (*S. cynipsea*: C; *S. neocynipsea*: N; and C × N versus N × C; female always named first) and biogeographical type nested within species (sympatry versus parapatry in Europe versus allopatry across continents) as fixed factors, and population nested within biogeographical type within species as a random effect. Certain pairings were additionally compared (planned comparisons): baseline behaviour of C versus N; cross-continental versus within-population N; and direction of heterospecific mating, i.e. C × N versus N × C. F_1 hybrid and backcrosses were analysed analogously but separately. We also performed two corresponding multivariate analyses subsuming, on the one hand, copulation frequency, male mating attempts and female shaking (using all data including zeroes) and, on the other hand, copulation latency and copulation duration (only for the subset of replicates in which copulations occurred).

A separate additional analysis to investigate the inheritance of all behavioural traits compared the conspecific parental pairings (N, C) with the F_1 hybrid offspring in both directions (C × N, N × C) using one-way univariate ANOVA with analogous nesting, fixed and random factors as above, followed by post hoc Tukey's tests. This qualitatively tested for deviations from the null expectation of intermediate inheritance, i.e. whether a trait shows dominance or maternal/paternal inheritance instead.

RESULTS

Mean values for all traits and pairings with 95% confidence intervals are reported in Tables 3 and 4. Detailed ANOVA statistics and covariate effects (*F* statistics, *P* values, β slopes) are reported in the Appendix.

Baseline Comparison of Conspecific Behaviour

Comparing the two species with four populations each as the baseline, *S. cynipsea* performed marginally more successful copulations per mating attempt than *S. neocynipsea* (Fig. 1a, Appendix Table A1). Lower copulation frequencies were associated with more male mating attempts (Appendix Table A1; no other covariate had a significant effect). This variation in copulation frequency reflects corresponding species differences in mating interactions, as *S. neocynipsea* males performed more mating attempts per 30 min than *S. cynipsea* males (Fig. 1b, Appendix Table A1), while *S. cynipsea* females displayed much more cumulative shaking (i.e. rejection or assessment behaviour: Fig. 1c, Appendix Table A1).

Table 3
Mean values (± 95% confidence interval) of all behavioural traits assessed

Generation	Pairing	Male mating attempts (No.)	Female shaking duration (min)	Copulation frequency (proportion)	Copulation latency (min)	Copulation duration (min)
Parental	C[a]	3.61±0.78	7.36±1.59	0.72±0.11	7.84±1.38	22.92±1.18
	C×N	5.04±1.31	3.27±0.88	0.09±0.05	11.04±2.63	16.92±3.04
	N×C	6.48±1.21	1.36±0.52	0.08±0.04	11.98±2.61	18.59±2.48
	N[a]	5.02±0.76	0.70±0.35	0.35±0.10	6.11±1.50	20.68±1.01
	N_{EU}[a]	4.53±0.96	0.86±0.64	0.37±0.15	6.99±2.29	20.46±1.78
	N×N	10.45±4.21	0.55±0.15	0.18±0.05	7.97±1.81	24.10±2.11
	N_{NA}[a]	5.49±1.15	0.54±0.30	0.31±0.13	4.84±1.77	21.11±1.68
F_1	C×N	5.04±0.14	4.03±1.34	0.45±0.11	7.71±1.72	23.33±1.72
	N×C	5.68±1.42	0.94±0.45	0.36±0.09	9.03±1.95	22.28±1.45
	N×N	8.57±1.45	0.42±0.12	0.34±0.07	6.95±1.70	24.71±0.99

C = *S. cynipsea*; N = *S. neocynipsea*; EU = Europe; NA = North America.
[a] Conspecific pairings: female × male.

Both traits stimulate each other as more male mating attempts necessarily entail more cumulative female shaking if the female is unwilling to mate (Appendix Table A1; for females affecting males $\beta = 0.136$, for males affecting females $\beta = 0.606$). The first copulation started somewhat earlier in *S. neocynipsea* than in *S. cynipsea* (Fig. 2a, Appendix Table A1), and copulation duration was slightly longer in *S. cynipsea* (Fig. 2b, Appendix Table A1).

Baseline Comparison of Intercontinental S. neocynipsea *Behaviour*

Copulation frequency (Fig. 1a) in cross-continental *S. neocynipsea* pairings did not vary between parental pairings, F_1 hybrids or backcrosses, nor did cumulative female shaking behaviour (Fig. 1c) and copulation latency (Fig. 2a). The only differences to be reported here are that males in cross-continental parental *S. neocynipsea* pairings performed more mating attempts than males in conspecific pairings within populations (Fig. 1b, Appendix Table A2). Likewise, males of the cross-continental F_1 (hybrid) generation performed more mating attempts than males in the conspecific parental pairings ($F_{1,6} = 13.59$, $P = 0.009$; Fig. 1b). In both comparisons, male mating attempts again covaried positively with female shaking (covariate effect: $F_{1,6} > 62.22$, $P < 0.001$, $\beta > 0.309$). Furthermore, copulation durations of F_1 hybrid pairings were longer than those of the parental conspecific pairings ($F_{1,6} = 15.39$, $P = 0.007$; Fig. 2b). Lastly, backcross direction showed no significant effect for any trait except copulation frequency ($F_{1,6} = 11.18$, $P = 0.012$), and was negatively affected by male mating attempts ($F_{1,53} = 25.491$, $P < 0.001$, $\beta = -0.556$) as well as female shaking duration ($F_{1,53} = 12.58$, $P = 0.001$, $\beta = -0.335$).

Heterospecific, F_1 Hybrid and Backcross Pairings

Heterospecific parental pairings never showed variation in crossing direction (C × N versus N × C) in any trait, except for copulation latency (Appendix Table A3). As expected, conspecific parental pairings resulted in much higher copulation frequencies than heterospecific pairings ($F_{3,12} = 18.01$, $P < 0.001$; Fig. 1a); copulation probability was additionally negatively related to the number of male mating attempts (Appendix Table A4). Even though there was only slight variation in the number of male mating attempts in an analogous test (Fig. 1b, Appendix Table A4), females in conspecific pairings showed longer cumulative shaking duration than those in heterospecific pairings (Fig. 1c, Appendix Table A4), the two traits again being correlated positively with each other (Appendix Table A4). Importantly, analogous multivariate analysis of all three traits together also indicated overall significant variation between species and cross types (C, N, C × N, N × C: $F_{9,36} = 13.19$, $P < 0.001$). Copulation latency was much longer and copulation duration significantly shorter in heterospecific pairings (Fig. 2a and b, Appendix Table A4). Again, the corresponding multivariate analysis was also significant ($F_{6,21} = 6.29$, $P < 0.001$).

Variation in F_1 hybrid direction (CxN versus NxC; grey versus orange dots in Fig. 1c) was only evident for female shaking duration, with less shaking observed when the mother was *S. neocynipsea* (Appendix Table A5). This comparison confirms the maternal inheritance of this trait (Fig. 3a), which is described in detail in the next section.

Backcrosses of F_1 hybrids with both parental species indicated no sex-specific variation for any of the studied behavioural traits, independent of whether the parental species was female or male (Appendix Figs A1, A2, Table A6).

Inheritance of Behavioural Traits to the F_1 Generation

Comparing the F_1 offspring, depending on hybrid direction (i.e. CxN versus NxC), with the parental species permits inferences about the inheritance of a trait (Fig. 3). A one-way GLM with pure

Table 4
Mean values (±95% confidence interval) of all behavioural traits assessed, regrouped by various criteria

Generation	Pairing	Male mating attempts (No.)	Female shaking duration (min)	Copulation frequency (proportion)	Copulation latency (min)	Copulation duration (min)
Parental	Conspecific	4.31±0.55	3.79±0.94	0.54±0.08	7.12±1.03	21.98±0.87
	Heterospecific	5.77±0.89	2.30±0.52	0.08±0.03	10.87±2.00	17.96±1.92
	Sympatry	4.69±1.14	1.00±0.58	0.04±0.04	13.00±6.06	18.75±5.97
	Parapatry	5.98±1.59	1.89±0.74	0.04±0.03	13.86±4.45	16.86±4.35
	Allopatry	6.69±1.83	3.91±1.12	0.16±0.07	11.21±2.26	18.02±2.20
F_1	Sympatry	5.11±1.52	3.46±1.46	0.42±0.15	8.77±2.58	24.51±2.18
	Parapatry	6.19±1.55	1.99±0.97	0.40±0.13	7.93±2.59	25.99±1.73
	Allopatry	5.17±1.65	1.96±1.24	0.44±0.11	8.56±1.90	20.79±1.41
Backcrosses	F_1×P	5.56±0.94	0.87±0.42	0.25±0.08	7.02±1.49	26.49±1.43
	P×F_1	5.75±0.85	1.26±0.56	0.27±0.09	8.41±2.12	25.00±1.94

Female × male.

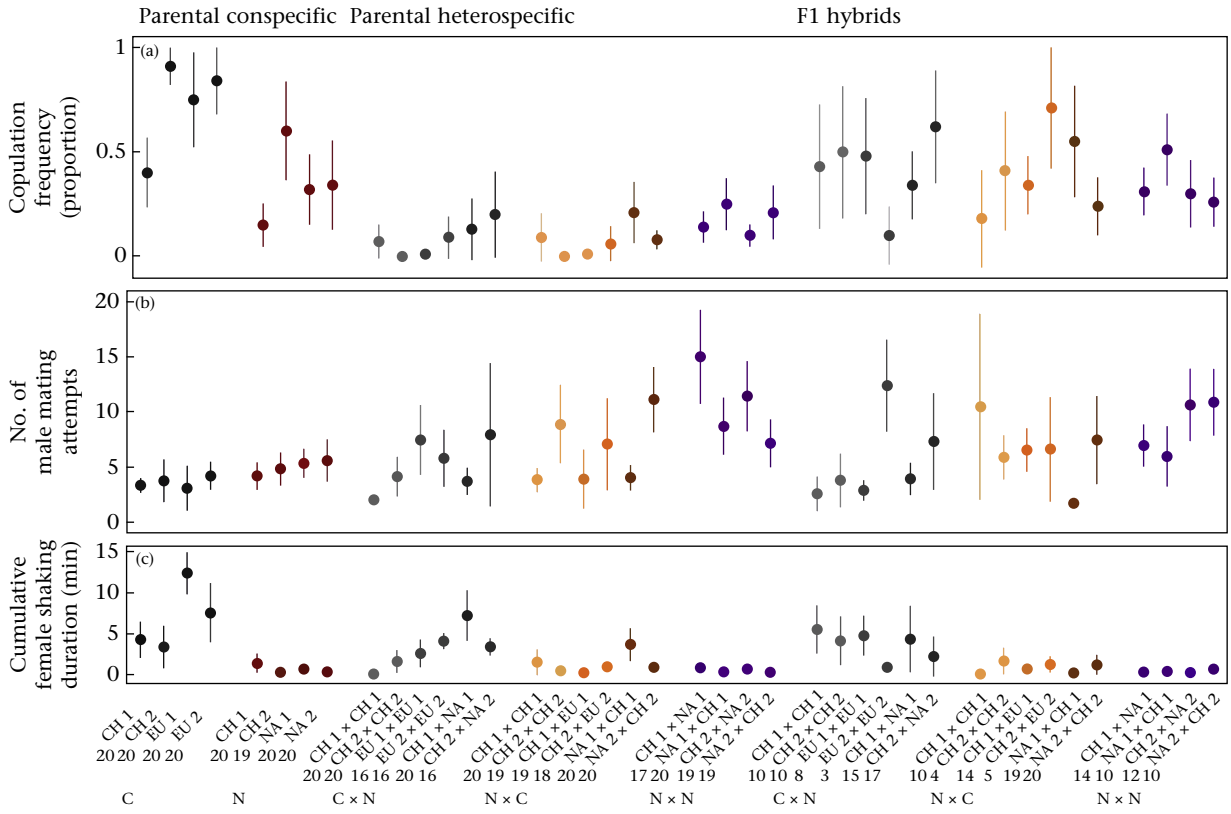

Figure 1. (a) Copulation frequency, (b) number of male mating attempts and (c) cumulative female shaking duration (population mean ± 95% confidence interval) for parental conspecific, heterospecific and F_1 hybrid offspring, representing all replicates (zeroes included). Conspecific parental pairings on the left are in black for *S. cynipsea* and red for *S. neocynipsea* populations. Heterospecific parental and F_1 hybrid pairings for C × N are in grey, N × C in orange and conspecific cross-continental *S. neocynipsea* (N × N) pairings in violet. Sympatric pairings are coloured lighter than parapatric and allopatric ones, the latter being darkest.

(baseline) parental pairings plus the reciprocal F_1 hybrids (CxN, NxC) as the main effect with four levels revealed significant variation for female shaking duration suggesting partial maternal inheritance ($F_{3,12} = 23.33$, $P < 0.001$; Fig. 3a); a post hoc Tukey's test further revealed significant differences between parental species (Appendix Table A7). An analogous one-way GLM further showed significant variation for copulation frequency suggesting dominance of *S. neocynipsea's* low copulation pattern ($F_{3,12} = 3.77$, $P = 0.038$; Fig. 3b, Appendix Table A7). All other traits showed no such variation suggesting the default intermediate inheritance (Appendix Fig. A3).

Effects of Biogeographical Type

Biogeographical type (sympatry, parapatry, allopatry) in the parental heterospecific pairings systematically affected copulation frequency, female shaking duration (Fig. 1a, c) and copulation latency (Fig. 2a), while the other traits showed no such variation. Copulation frequency and shaking duration increased from sympatric via parapatric to allopatric cross-continental pairings (Fig. 1a, Appendix Table A4). Similarly, flies of the cross-continental allopatric parental pairings required less time until the first copulation ensued than flies from the corresponding European parapatric and sympatric pairings, although this appeared to be the case primarily in the CxN but not the NxC subset of the data (Fig. 2a, Appendix Table A4). Crucially, the multivariate analysis subsuming all the traits presented in Fig. 1 also yielded a significant effect of biogeographical type ($F_{12,18} = 3.49$, $P = 0.008$), which was not the case for the two copulation traits presented in Fig. 2 ($F_{8,8} = 3.27$, $P = 0.057$).

Analogous analysis of the F1 hybrid generation only revealed systematic effects of biogeographical type on female shaking, with a decrease in shaking duration from sympatric to allopatric pairings (Fig. 1c, Appendix Table A1), but not on any other traits.

DISCUSSION

Sepsis cynipsea and *S. neocynipsea* have been described as different species based on their mitochondrial genetic distances (Puniamoorthy et al., 2008; Su et al., 2008), as well as behavioural and morphological differences (Pont & Meier, 2002). The species show low conspecific population differentiation but high heterospecific genetic differentiation based on neutral genetic microsatellite markers (Baur et al., 2017). Furthermore, North American and European populations of *S. neocynipsea* were recognized as the same species despite their geographical isolation and some morphological differences (Pont & Meier, 2002). We here documented quantitative differences in some precopulatory behavioural traits important for mating that are shared by both species, notably male mating attempts, female shaking behaviour, copulation frequency, latency and duration. Female shaking when males are mounted on their back, a trait that is part of the general repertoire of sepsid flies (Puniamoorthy et al., 2009), is much more pronounced in *S. cynipsea* than in *S. neocynipsea*. Previous studies of *S. cynipsea* had identified this trait as contributing to female choice of mating partners and/or an expression of female reluctance to mate (Blanckenhorn et al., 2000; Parker, 1972a, 1972b; Ward, 1983; Ward et al., 1992); it also has been shown to evolve in response to mating system manipulations due to sexual selection and conflict (Martin & Hosken, 2003).

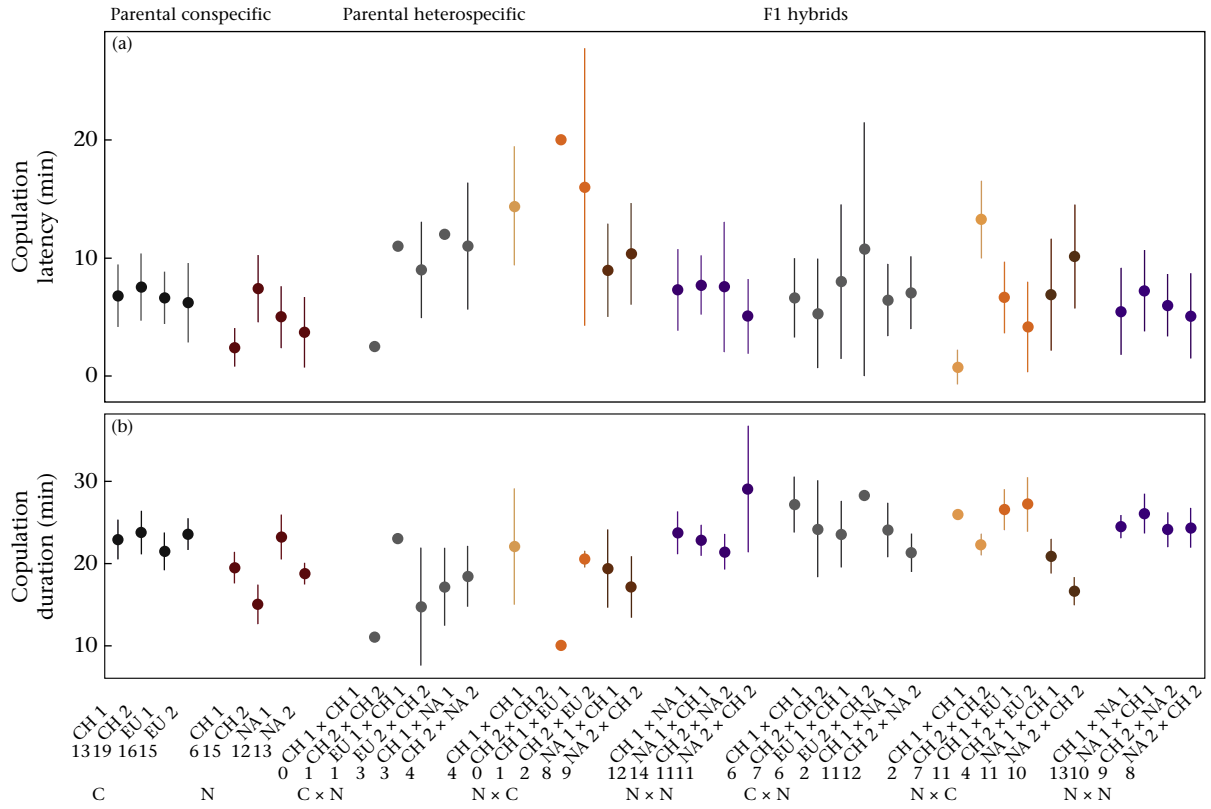

Figure 2. (a) Copulation latency and (b) copulation duration (population mean ± 95% confidence interval) for parental conspecific, heterospecific and F_1 hybrid offspring, representing only replicates with at least one copulation. Conspecific parental pairings on the left are in black for *S. cynipsea* and red for *S. neocynipsea* populations. Heterospecific parental and F1 hybrid pairings for C × N are in grey, N × C in orange and conspecific cross-continental *S. neocynipsea* pairings (N × N) in violet. Sympatric pairings are coloured lighter than parapatric and allopatric ones, the latter being darkest.

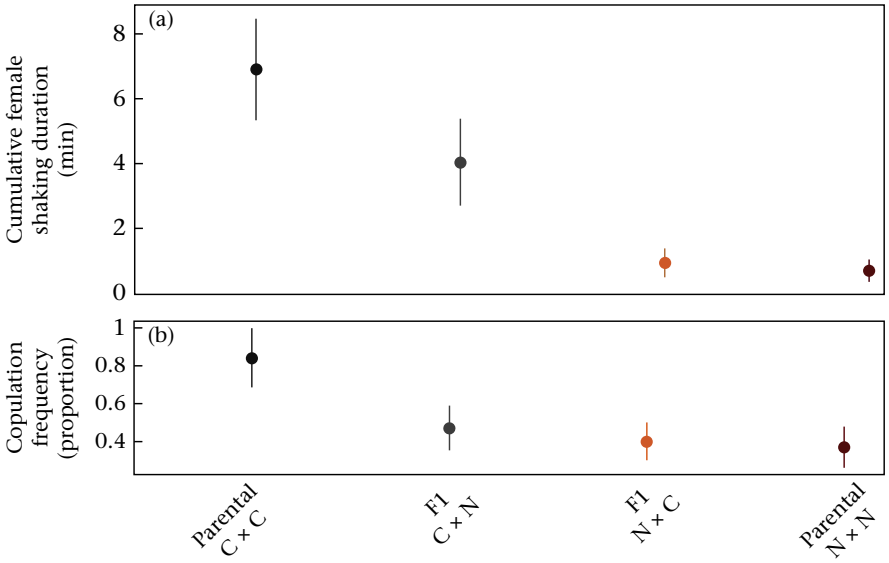

Figure 3. (a) Cumulative female shaking duration (indicating maternal inheritance) and (b) copulation frequency (indicating dominance) compared between conspecific *S. cynipsea* (black), *S. neocynipsea* (red) and F_1 hybrid offspring (grey for C × N, orange for N × C) pairings for evaluating the inheritance pattern of behavioural traits (mean ± 95% confidence interval).

We were here able to detect that females, across almost all pairings and generations, responded with higher cumulative shaking to more male mating attempts, but this resulted in lower copulation frequencies, indicating reluctance to mate (Blanckenhorn et al., 2000). However, *S. cynipsea* females did shake longer in conspecific pairings than *S. neocynipsea* females resulting in higher copulation frequencies, suggesting mate assessment. Overall, if the female is willing to mate, males do not attempt to mount her so often, in turn indicating her willingness to copulate. Nevertheless, our evidence here that female shaking contributes to

mate recognition, which could lead to reproductive isolation, is limited to significant variation in the expected direction between sympatric, parapatric and allopatric heterospecific pairings (Fig. 1c). It should be clear that traits merely differing quantitatively do not serve as well for reproductive isolation as do qualitatively different courtship traits (Puniamoorthy, 2014). Overall, our study revealed strong evidence for possible hybridization of these two species, and also some evidence of species recognition and reproductive isolation at the precopulatory level, most apparent in terms of lower copulation frequencies and, particularly, in longer copulation latencies in heterospecific pairings. Under laboratory conditions, viable F_1 hybrid offspring had higher copulation success with each other and even with parental partners in backcrosses than occurred in the baseline conspecific parental pairings. This indicates hybrid vigour rather than outbreeding depression, facilitating hybridization in nature (Todesco, Pascual, Owens, 2016; Wolf, Takebayasi, & Rieseberg, 2001). We are currently investigating at the genomic level the extent to which hybridization occurs in nature.

Comparison of Parental Behaviour

Our study revealed anticipated but so far not quantified differences between the sister species in most assessed traits (Fig. 1), verifying on behavioural grounds that the two species are indeed separate but very closely related (Pont & Meier, 2002; Puniamoorthy et al., 2008; Su et al., 2008). Besides the prominent differences in female shaking behaviour discussed above, species differences in the other four traits assessed here are less pronounced. *Sepsis neocynipsea* males exhibited more mating attempts than *S. cynipsea* males. In nature, *S. cynipsea* is much more abundant than *S. neocynipsea* (and any other sepsid species) in places of co-occurrence in Europe, such as the Swiss Alps from where we sampled our populations (Pont & Meier, 2002). This implies that *S. cynipsea* males should more easily find conspecific mating partners, and therefore do not need to try hard to achieve copulations. Despite more potential harassment of *S. neocynipsea* females by males of the other species in nature, known as the rare female hypothesis (Noor, 1995; Yukilevich, 2012), the latter performed less shaking but nevertheless ended up maintaining lower copulation frequencies, indicating that whatever other means *S. neocynipsea* females use to fend off unwanted males are very effective. *Sepsis cynipsea* females, in contrast, showed stronger shaking; nevertheless this resulted in more copulations per mating attempt, re-emphasizing the role of this behaviour in mate assessment (Ward, 1983).

Male and female traits depended significantly on each other, showing that the cumulative female shaking duration was longer the more often males attempted to mate with a partner, most likely to fend off the constant harassment by males. In turn, copulation frequency depended significantly on male–female interactions. Copulation success per mating attempt was lower in *S. neocynipsea*, for which ca. 35% of all male mating attempts resulted in copulations, as opposed to ca. 72% for *S. cynipsea* (Table 3). We recorded more copulations in conspecific pairings when males needed fewer attempts, probably because females seemed to be more willing to mate, possibly facilitated by species recognition of the conspecific partner. As expected, copulation success in our forced heterospecific pairings was much lower (ca. 8%). Moreover, conspecific *S. cynipsea* pairings showed longer copulation latencies, suggesting *S. cynipsea* females spend overall more time assessing mates (by shaking more; Blanckenhorn et al., 2000), whereas *S. neocynipsea* females started copulating faster when mounted by a conspecific male. Finally, *S. cynipsea* showed slightly longer copulation durations (ca. 23 versus 21 min), the biological significance of which is probably minor.

Perhaps surprisingly, *S. cynipsea* females shook less in heterospecific than conspecific pairings (Table 4). This lower shaking duration when facing heterospecific males could be a result of faster male dismounting or dislocation due to the species differences in the male armoured foreleg, which is an important male tool to cling on to the female's wing (Pont & Meier, 2002). In this context shaking appears effective for *S. cynipsea* females in assessing or rejecting mates (Blanckenhorn et al., 2000, 1999; Martin & Hosken, 2003; Ward, 1983; Ward et al., 1992), while *S. neocynipsea* females must have other means of assessing unwanted males: for instance, surface or volatile hydrocarbons could be involved (Puniamoorthy, 2013). Other candidates could be subtle male courtship behaviour (e.g. circling around a female) or leg positions during pairing, which have been demonstrated in several sepsid species and many other insects (Eberhard, 1996; Puniamoorthy et al., 2009). Species recognition in heterospecific pairings here is most prominently expressed in longer copulation latencies than in conspecific pairings. The lower copulation frequencies, shorter copulation durations and longer copulation latencies in heterospecific pairings all signify strongly that these flies have more difficulties in mating, probably due to divergence in mate recognition systems.

Sepsis neocynipsea males in cross-continental, conspecific parental pairings performed more mating attempts, while not eliciting more female shaking, perhaps because they dismounted faster on their own. On the other hand, similar female shaking durations, copulation frequencies and latencies, with only minor differences in the other behavioural traits between the continents and across all generations, indicate that *S. neocynipsea* from North America and Europe indeed recognize each other as the same species.

Trait Inheritance in F_1 Hybrids and Hybrid Vigour

Our study revealed no significant variation related to hybrid direction (C × N versus N × C) for any behavioural trait, except for female shaking behaviour. Accordingly, F_1 hybrid offspring showed intermediate phenotypes relative to the parental species, indicating intermediate and presumably mainly autosomal inheritance of most of the quantitative behavioural traits considered here. Shaking behaviour is a prominent exception, which appears to be at least partly maternally inherited because hybrids expressed shaking more similar to the maternal species (Fig. 3a). Copulation frequency further showed evidence for dominance, as the lower copulation probabilities of *S. neocynipsea*, mediated by whatever mechanism, seemed to be inherited by all the hybrids (Fig. 3b). Although our study design was not suited to calculate heritabilities, we were able to detect these strong signs of maternal inheritance and dominance. Further work to explore the genetic basis underlying these mechanisms could be a central aim of future studies. Our results confirm that most mating traits considered here are quantitative and heritable, and can therefore evolve in response to natural and sexual selection (see Martin & Hosken, 2003; Mühlhäuser & Blanckenhorn, 2004).

Interestingly, hybrid vigour was evident in F_1 hybrid offspring, not so much for the male and female behavioural traits themselves, but certainly by virtue of the increased copulation success relative to the heterospecific parental pairings (Fig. 1, Tables 3, 4; Baranwal, Mikkilineni, Zehr, Tyagi, & Kapoor, 2012). We can therefore conclude that hybridization does not immediately lead to cessation of mating behaviour and copulation in this system, although this may happen in later generations or further backcrosses. A first sign of mating barriers may be increased copulation durations of hybrids, suggesting some postmating but prezygotic difficulties such as disturbed sperm transfer (Arthur & Dyer, 2015). We will

investigate in future studies whether reproductive success is depressed in hybrids or backcrosses despite the continuing mating success documented here.

Comparing Generations: Are Hybrids Recognized?

Our results revealed little variation in most traits across the generations (parental, F_1, backcrosses), highlighting no breakdown over generations of important traits that potentially could reduce mating success. Instead, hybridization may merely be disrupted by the difficulties in sperm transfer indicated by prolonged copulation durations of F_1 hybrids. Invariant male mating attempts also indicate that males do not discriminate strongly against heterospecific partners, as can be expected because sperm are relatively cheap, whereas the effort put into achieving a mating is substantial (Birkhead & Møller, 1998). The mating system of both species is best described as scramble competition, with few if any aggressive interactions among males and a paramount role of female choice, by whatever mechanism (Blanckenhorn et al., 2000).

Comparing Across the Biogeographical Range

The strongest precopulatory isolation is often demonstrated in sympatric species pairs, indicating reinforcement (Coyne & Orr, 1989, 2004; Yukilevich, 2012). A biogeographical effect in the parental pairings was detected for copulation frequency, latency and female shaking behaviour (Figs. 1 and 2). Heterospecific pairings from European populations in either sympatry or parapatry exhibited longer latencies to copulation than flies in the cross-continental allopatric pairings, suggesting reinforcement of species recognition in areas where the two species co-occur. High conspecific gene flow may maintain this pattern throughout Europe (Fig. 1, Table 4). In contrast, heterospecific parental pairings showed stronger female shaking in allopatric pairings across continents and little shaking in sympatric pairings, although this pattern may be equally explained by faster dismounting of unwanted mates and species recognition of males in sympatric pairings. Reinforcement through stronger female shaking behaviour in areas of sympatry is also reflected in the F_1 hybrid offspring, for which sympatric pairings showed more shaking than allopatric pairings (Fig. 1, Table 4).

Conclusions

We documented successful hybridization under forced laboratory conditions between the close sister species *S. cynipsea* and *S. neocynipsea*. Female mate choice and species recognition can explain the low frequency of heterospecific relative to conspecific copulations realized per male mating attempt as well as their longer copulation latencies. The observed pattern of F_1 hybrids and backcrosses showing lower copulation frequencies, longer copulation latencies and durations than the conspecific parental pairings, while at the same time achieving more copulations than flies in heterospecific pairings, could result from hybrid vigour mediated by a mixture of genes from both species permitting species recognition (Baranwal et al., 2012). We also observed heterospecific parental pairings with lower, and F_1 and backcross pairings with higher, copulation durations than the parental species, indicating possible difficulties with sperm transfer. Copulations do not necessarily imply successful fertilization, however, so offspring production needs to be documented to reveal possible mechanisms of postcopulatory isolation such as male sterility (Haldane, 1922).

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Supplementary material

Supplementary material associated with this article is available, in the online version, at http://dx.doi.org/10.1016/j.anbehav.2017.08.008.

References

Arthur, N. J., & Dyer, K. A. (2015). Asymmetrical sexual isolation but no postmating isolation between the closely related species *Drosophila suboccidentalis* and *Drosophila occidentalis*. *BioMed Central Evolutionary Biology, 15*(38). http://dx.doi.org/10.1186/s12862-015-0328-y.

Baranwal, V. K., Mikkilineni, V., Zehr, U. B., Tyagi, A. K., & Kapoor, S. (2012). Heterosis: Emerging ideas about hybrid vigour. *Journal of Experimental Botany, 63*(18), 6309–6314. http://dx.doi.org/10.1093/jxb/ers/291.

Baur, J., Schäfer, M. A., Blanckenhorn, W. U., & Giesen, A. (2017). *Patterns of genetic differentiation in sexual and neutral morphological traits among species and populations of dung flies (Diptera: Sepsidae)*. Unpublished manuscript.

Birkhead, T. R., & Møller, A. P. (1998). *Sperm competition and sexual selection*. San Diego, CA: Academic Press.

Blanckenhorn, W. U., Morf, C., Mühlhäuser, C., & Reusch, T. (1999). Spatio-temporal variation in selection on body size in the dung fly *Sepsis cynipsea*. *Journal of Evolutionary Biology, 12*, 563–576. http://dx.doi.org/10.1046/j.1420-9101.1999.00050.x.

Blanckenhorn, W. U., Mühlhäuser, C., Morf, C., Reusch, T., & Reuter, M. (2000). Female choice, female reluctance to mate and sexual selection on body size in the dung fly *Sepsis cynipsea*. *Ethology, 106*, 577–593. http://dx.doi.org/10.1046/j.1439-0310.2000.00573.x.

Boake, C. R. B., Price, D. K., & Andreadis, D. K. (1998). Inheritance of behavioral differences between two interfertile, sympatric species *Drosophila silvestris* and *D. heteroneura*. *Heredity, 80*, 642–650.

Coyne, J. A., & Orr, H. A. (1989). Patterns of speciation in *Drosophila* revisited. *Evolution, 51*, 295–303. http://dx.doi.org/10.2307/2410984.

Coyne, J. A., & Orr, H. A. (2004). *Speciation*. Sunderland M.A: Sinauer.

Ding, A., & Blanckenhorn, W. U. (2002). The effect of sexual size dimorphism on mating behavior in two dung flies with contrasting dimorphism. *Evolutionary Ecology Resources, 4*, 259–273.

Dobzhansky, T. (1951). *Genetics and the origin of species* (3rd ed.). New York N.Y.: Columbia University Press.

Eberhard, W. G. (1996). *Female control: Sexual selection by cryptic female choice*. Princeton, N.J.: Princeton University Press.

Eberhard, W. G. (1999). Mating systems of sepsid flies and sexual behavior away from oviposition sites by *Sepsis neocynipsea* (Diptera: Sepsidae). *Journal of the Kansas Entomological Society, 72*(1), 129–130.

Gavrilets, S. (2000). Rapid evolution of reproductive barriers driven by sexual conflict. *Nature, 403*, 886–889. http://dx.doi.org/10.1038/35002564.

Gleason, J. M., & Ritchie, M. G. (1998). Evolution of courtship song and reproductive isolation in the *Drosophila willistoni* species complex: Do sexual signals diverge the most quickly? *Evolution, 52*, 1493–1500. http://dx.doi.org/10.2307/2411319.

Haldane, J. B. S. (1922). Sex ratio and the unisexual sterility in animal hybrids. *Journal of Genetics, 12*, 101–109. http://dx.doi.org/10.1007/BF02983075.

Hood, G. R., Egan, S. P., & Feder, J. L. (2012). Evidence for sexual isolation as a prezygotic barrier to gene flow between morphologically divergent species of *Rhagoletis* fruit flies. *Ecological Entomology, 37*(6), 521–528. http://dx.doi.org/10.1111/j.1365-2311.2012.01392.x.

Hosken, D. J., Martin, O. Y., Born, J., & Huber, F. (2003). Sexual conflict in *Sepsis cynipsea*: Female reluctance, fertility and mate choice. *Journal of Evolutionary Biology, 16*(3), 485–490.

Kozak, G. M., Reisland, M., & Boughmann, J. W. (2009). Sex differences in mate recognition and conspecific preference in species with mutual mate choice. *Evolution, 63*, 353–365. http://dx.doi.org/10.1111/j.1558-5646.2008.00564.x.

Kraaijeveld, K., Kraaijeveld-Smit, F. J. L., & Maan, M. E. (2010). Sexual selection and speciation: The comparative evidence revisited. *Biological Reviews, 86*(2), 367–377. http://dx.doi.org/10.1111/j.1469-185X.2010.00150.x.

Lande, R. (1981). Model of speciation by sexual selection on polygenic traits. *Proceedings of the National Academy of Sciences of the United States of America, 78*(6), 3721–3725.

Martin, O. Y., & Hosken, D. J. (2003). The evolution of reproductive isolation through sexual conflict. *Nature, 423*, 979–982. http://dx.doi.org/10.1038/nature01752.
Mayr, E. (1942). *Systematics and the origin of species*. New York, N.Y.: Columbia University Press.
Mühlhäuser, C., & Blanckenhorn, W. U. (2004). The quantitative genetics of sexual selection in the dung fly *Sepsis cynipsea*. *Behaviour, 141*(3), 327–341. http://dx.doi.org/10.1163/156853904322981888.
Noor, M. A. (1995). Speciation driven by natural selection *Drosophila*. *Nature, 375*, 674–675. http://dx.doi.org/10.1038/375674a0.
Ozerov, A. L. (2005). World catalogue of the family Sepsidae (insect: Diptera). *Zoologicheskie Issledovania (Zoological Studies), 8*, 1–74.
Panhuis, T. M., Butlin, R., Zuk, M., & Tregenza, T. (2001). Sexual selection and speciation. *Trends in Ecology & Evolution, 16*(7), 364–371. http://dx.doi.org/10.1016/S0169-5347(01)02160-7.
Parker, G. A. (1972a). Reproductive behaviour of *Sepsis cynipsea* (L.) (Diptera: Sepsidae). I. A preliminary analysis of the reproductive strategy and its associated behaviour patterns. *Behaviour, 41*, 172–206. http://dx.doi.org/10.1163/156853972X00257.
Parker, G. A. (1972b). Reproductive behaviour of *Sepsis cynipsea* (L.) (Diptera: Sepsidae) II. The significance of the precopulatory passive phase and emigration. *Behaviour, 41*, 242–250. http://dx.doi.org/10.1163/156853972X00031.
Pont, A. C., & Meier, R. (2002). *The Sepsidae (Diptera) of Europe*. Leiden, The Netherlands: Brill.
Puniamoorthy, N. (2013). *Morphology, molecules and mating behavior: An integrative study of population divergence and speciation in widespread sepsid flies (Sepsidae: Diptera)* (Doctoral thesis). Zurich, Switzerland: University of Zurich.
Puniamoorthy, N. (2014). Behavioural barriers to reproduction may evolve faster than sexual morphology among populations of a dung fly (Sepsidae). *Animal Behaviour, 98*, 139–148. http://dx.doi.org/10.1016/j.anbheav.2014.10.005.
Puniamoorthy, N., Ismail, M. R. B., Tan, D. S. H., & Meier, R. (2009). From kissing to belly stridulation: Comparative analysis reveals surprising diversity, rapid evolution, and much homoplasy in the mating behaviour of 27 species of sepsid flies (Diptera: Sepsidae). *Journal of Evolutionary Biology, 22*(11), 2146–2156. http://dx.doi.org/10.111/j.1420-9101.2009.01826.x.
Puniamoorthy, N., Schäfer, M. A., & Blanckenhorn, W. U. (2012). Sexual selection accounts for the geographic reversal of sexual size dimorphism in the dung fly *Sepsis punctum* (Diptera: Sepsidae). *Evolution, 66*(7), 2117–2126. http://dx.doi.org/10.1111/j.1558-5646.2012.01599.x.
Puniamoorthy, N., Su, K. F. Y., & Meier, R. (2008). Bending for love: Losses and gains of sexual dimorphisms are strictly correlated with changes in the mounting position of sepsid flies (Sepsidae: Diptera). *BMC Evolutionary Biology, 8*(1), 155. http://dx.doi.org/10.1186/1471-2148-8-155.
Reed, L. K., & Markow, T. A. (2004). Early events in speciation: Polymorphism for hybrid male sterility in *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America, 101*, 9009–9012. http://dx.doi.org/10.1073/pnas.0403106101.
Rohner, P. T., Blanckenhorn, W. U., & Puniamoorthy, N. (2016). Sexual selection on male size drives the evolution of male-biased sexual size dimorphism via the prolongation of male development. *Evolution, 70*(6), 1189–1199. http://dx.doi.org/10.1111/evo.12944.
Schluter, D. (2000). *The ecology of adaptive radiation*. Oxford, U.K.: Oxford University Press.
Schluter, D. (2001). Ecology and the origin of species. *Trends in Ecology and Evolution, 16*, 372–380. http://dx.doi.org/10.1016/S0169-5347(01)02198-X.
Shaw, K. L., & Herlihy, D. P. (2000). Acoustic preference functions and song variability in the Hawaiian cricket *Laupala cerasina*. *Proceedings of the Royal Society B: Biological Sciences, 267*, 577–584. http://dx.doi.org/10.1098/rspb.2000.1040.
Su, K. F. Y., Kutty, S., & Meier, R. (2008). Morphology versus molecules: The phylogenetic relationships of Sepsidae (Diptera: Cyclorrhapha) based on morphology and DNA sequence data from ten genes. *Cladistics, 24*, 902–916. http://dx.doi.org/10.1111/j.1096-0031.2008.00222.x.
Svensson, E. I., Karlsson, K., Friber, M., & Eroukhmanoff, F. (2007). Gender differences in species recognition and the evolution of asymmetric sexual isolation. *Current Biology, 17*, 1943–1947. http://dx.doi.org/10.1016/j.cub.2007.09.038.
Teuschl, Y., & Blanckenhorn, W. U. (2007). The reluctant fly: What makes *Sepsis cynipsea* females willing to copulate? *Animal Behaviour, 73*, 85–97. http://dx.doi.org/10.1016/j.anbehav.2006.03.028.
Todesco, M., Pascual, M. A., Owens, G. L., Ostevik, K. L., Moyers, B. T., Hübner, S., et al. (2016). Hybridization and extinction. *Evolutionary Applications, 9*(7), 892–908. http://dx.doi.org/10.1111/eva.12367.
Turelli, M., Barton, N. H., & Coyne, J. A. (2001). Theory and speciation. *Trends in Ecology & Evolution, 16*(7), 330–343. http://dx.doi.org/10.1016/S0169-5347(01)02177-2.
Via, S. (2001). Sympatric speciation in animals: The ugly duckling grows up. *Trends in Ecology and Evolution, 16*, 381–390. http://dx.doi.org/10.1016/S0169-5347(01)02188-7.
Via, S. (2009). Natural selection in action during speciation. *Proceedings of the National Academy of Sciences of the United States of America, 106*, 9939–9946. http://dx.doi.org/10.1073/pnas.0901397106.
Ward, P. I. (1983). The effects of size on the mating behaviour of the dung fly *Sepsis cynipsea*. *Behavioral Ecology and Sociobiology, 13*, 75–80. http://dx.doi.org/10.1007/BF00295078.
Ward, P. I., Hemi, J., & Rösli, T. (1992). Sexual conflict in the dung fly *Sepsis cynipsea*. *Functional Ecology, 6*, 649–653.
Wassermann, M., & Koepfer, H. R. (1977). Character displacement for sexual isolation between *Drosophila mojavensis* and *Drosophila arizonensis*. *Evolution, 31*(4), 812–823. http://dx.doi.org/10.2307/2407442.
Wolf, D. E., Takebayasi, N., & Rieseberg, L. H. (2001). Predicting the risk of extinction through hybridization. *Conservation Biology, 15*(4), 1039–1053. http://dx.doi.org/10.1046/j.1523-1739.2001.0150041039.x.
Yukilevich, R. (2012). Asymmetrical patterns of speciation uniquely support reinforcement in *Drosophila*. *Evolution, 66*, 1430–1446. http://dx.doi.org/10.1111/j.1558-5646.2011.01534.

Appendix

Table A1
ANOVA between parental *S. cynipsea* and *S. neocynipsea*

Trait	*df*	*F*	*P*	β
No. of male mating attempts	1, 6	9.641	**0.021**	
With female shaking duration	1, 144	12.900	**<0.001**	0.136
Female shaking duration	1, 6	33.727	**0.001**	
With male mating attempts	1, 144	12.900	**<0.001**	0.606
Copulation frequency	1, 6	6.437	**0.044**	
With male mating attempts	1, 150	12.281	**0.001**	−0.365
Copulation latency	1, 6	5.356	***0.055***	
Copulation duration	1, 6	4.475	***0.073***	

Bold values indicate $P < 0.05$; values in italics indicate $P < 0.1$. Baseline planned subcomparison of all behavioural traits between parental conspecific *S. cynipsea* and *S. neocynipsea* by univariate analyses of variance. Only significant covariates are reported.

Table A2
ANOVA between continental (EU versus NA) pairings and cross-continental (N × N) versus within-continental pairings of *S. neocynipsea*

Comparison	Trait	*df*	*F*	*P*	β
EU versus NA	No. of male mating attempts	1, 2	5.969	0.134	
	Female shaking duration	1, 2	0.108	0.773	
	Copulation frequency	1, 2	0.040	0.860	
	Copulation latency	1, 2	0.160	0.727	
	Copulation duration	1, 2	0.109	0.772	
Cross- versus within-continental	No. of male mating attempts	1, 6	12.085	**0.013**	
	With female shaking duration	1, 142	62.985	**<0.001**	+0.297
	Female shaking duration	1, 6	2.065	0.201	
	With male mating attempts	1, 142	62.985	**<0.001**	+0.446
	Copulation frequency	1, 6	3.037	0.132	
	Copulation latency	1, 6	2.172	0.188	
	Copulation duration	1, 6	4.616	0.073	

Bold values indicate $P < 0.05$; values in italics indicate $P < 0.1$. Comparison of parental conspecific *S. neocynipsea* pairings across continents (EU versus NA), and of cross-continental (N × N) versus within-continental pairings by univariate analyses of variance for all behavioural traits.

Table A3
ANOVA between parental heterospecific pairings

Trait	*df*	*F*	*P*	β
No. of male mating attempts	1, 6	0.502	0.505	
Female shaking duration	1, 6	4.533	***0.077***	
Copulation frequency	1, 6	0.077	0.791	
Copulation latency	1, 4	6.933	**0.013**	
Copulation duration	1, 6	0.260	0.621	

Bold values indicate $P < 0.05$; values in italics indicate $P < 0.1$. Planned subcomparison of all behavioural traits according to the direction of heterospecific parental pairing (C × N versus N × C) by nested univariate analyses of variance. No significant covariates.

Table A4
ANOVA comparing conspecific and heterospecific parental pairings and nested biogeographical types

Comparison	Trait	*df*	*F*	*P*	β
C, N, C×N, N×C	No. of male mating attempts	3, 12	3.32	***0.055***	
	With female shaking duration	1, 349	32.752	**<0.001**	+0.158
	Female shaking duration	3, 12	13.047	**0.001**	
	With male mating attempts	1, 349	32.752	**<0.001**	+0.542
	Copulation frequency	3, 12	21.488	**<0.001**	
	With male mating attempts	1, 362	18.001	**<0.001**	−0.186
	Copulation latency	3, 10	9.462	**<0.001**	
	Copulation duration	3, 11	5.663	**0.006**	
Biogeographical type	No. of male mating attempts	4, 6	0.619	0.665	
	Female shaking duration	4, 6	4.969	**0.041**	
	Copulation frequency	4, 6	11.782	**0.005**	
	Copulation latency	4, 4	5.844	**0.001**	
	Copulation duration	4, 5	0.811	0.522	

Bold values indicate $P < 0.05$; values in italics indicate $P < 0.1$. Univariate analyses of variance comparing conspecific and heterospecific parental pairings and nested biogeographical type (sympatry, parapatry, allopatry) for all behavioural traits (cross-continental *S. neocynipsea* pairings excluded). Only significant covariates are reported. Error degrees of freedom for the copulation traits are lower because there were no copulations in some replicates.

Table A5
ANOVA between heterospecific F1 hybrid directions and biogeographical type

Comparison	Trait	*df*	*F*	*P*	β
C×N versus N×C	No. of male mating attempts	1, 6	1.178	0.311	
	With female shaking duration	1, 88	6.405	**0.013**	+0.134
	Female shaking duration	1, 6	21.821	**0.001**	
	With male mating attempts	1, 88	6.405	**0.013**	+0.507
	Copulation frequency	1, 6	0.452	0.512	
	Copulation latency	1, 6	0.214	0.657	
	Copulation duration	1, 6	1.099	0.325	
Biogeographical type	No. of male mating attempts	4, 6	1.370	0.342	
	Female shaking duration	4, 6	3.349	***0.075***	
	Copulation frequency	4, 6	3.137	***0.072***	
	Copulation latency	4, 6	0.271	0.887	
	Copulation duration	4, 6	4.480	**0.042**	

Bold values indicate $P < 0.05$; values in italics indicate $P < 0.1$. Univariate analyses of variance comparing hybrid F1 pairings and nested biogeographical type (sympatry, parapatry, allopatry) for all behavioural traits (cross-continental *S. neocynipsea* pairings excluded). Only significant covariates are reported.

Table A6
ANOVA between all heterospecific backcross directions

Comparison	Trait	*df*	*F*	*P*
All backcross types	No. of male mating attempts	7, 16	1.728	0.163
	Female shaking duration	7, 12	1.574	0.216
	Copulation frequency	7, 16	1.003	0.459
	Copulation latency	7, 10	1.600	0.223
	Copulation duration	7, 10	1.521	0.243
P×F_1 versus F_1×P	No. of male mating attempts	1, 6	1.008	0.328
	Female shaking duration	1, 6	2.616	0.139
	Copulation frequency	1, 6	0.063	0.805
	Copulation latency	1, 6	1.256	0.287
	Copulation duration	1, 6	0.931	0.357

Bold values indicate $P < 0.05$; values in italics indicate $P < 0.1$. Comparison of all behavioural traits for all backcross types and backcross direction (P × F_1 versus F_1 × P) by univariate analyses of variance.

Table A7
Post hoc paired Tukey's tests evaluating the inheritance pattern of behavioural traits

Species	P C	F_1 C×N	F_1 N×C	P N
P C	–	0.262±0.132	0.992±0.128	1.205±0.115
		P=0.195	*P*<0.001	*P*<0.001
F_1 C×N	−0.270±0.077	–	0.730±0.142	0.943±0.131
	P=0.003		*P*<0.001	*P*<0.001
F_1 N×C	−0.326±0.074	−0.056±0.078	–	0.213±0.127
	P<0.001	*P*=0.891		*P*=0.986
P N	−0.373±0.072	−0.103±0.077	−0.470±0.074	–
	P<0.001	*P*=0.537	*P*=0.921	

C = *S. cynipsea*; N = *S. neocynipsea*; female × male: C × N, N × C. Post hoc paired Tukey's tests followed the one-way ANOVA comparing parental (P) conspecific (C, N) and F_1 hybrid pairings for evaluating the inheritance pattern of behavioural traits, calculated as the difference between the pairings ± SEs. Female shaking duration (min) is shown above and copulation frequency below the diagonal.

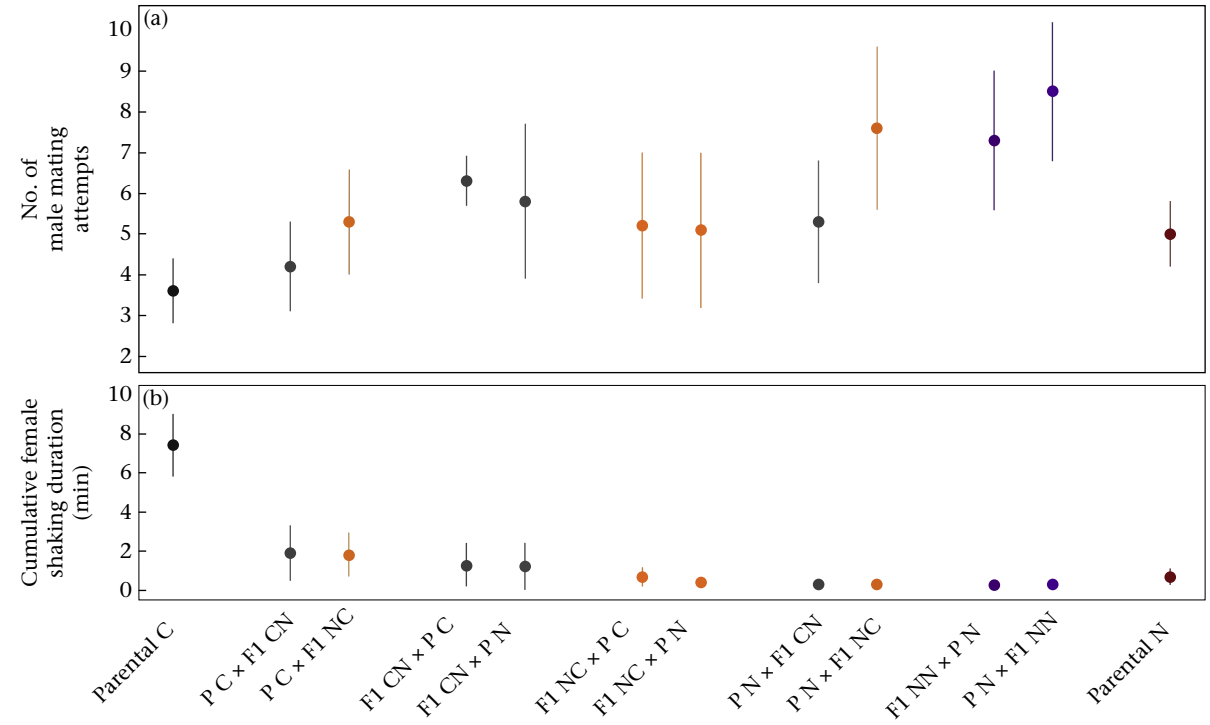

Figure A1. Comparison of male and female behavioural traits of parental and backcross pairings (mean ± 95% confidence interval). (a) Number of male mating attempts and (b) cumulative female shaking duration. Backcrosses with C × N hybrids are in grey, those with N × C hybrids in orange. Continental *S. neocynipsea* (N × N) backcrosses are in violet.

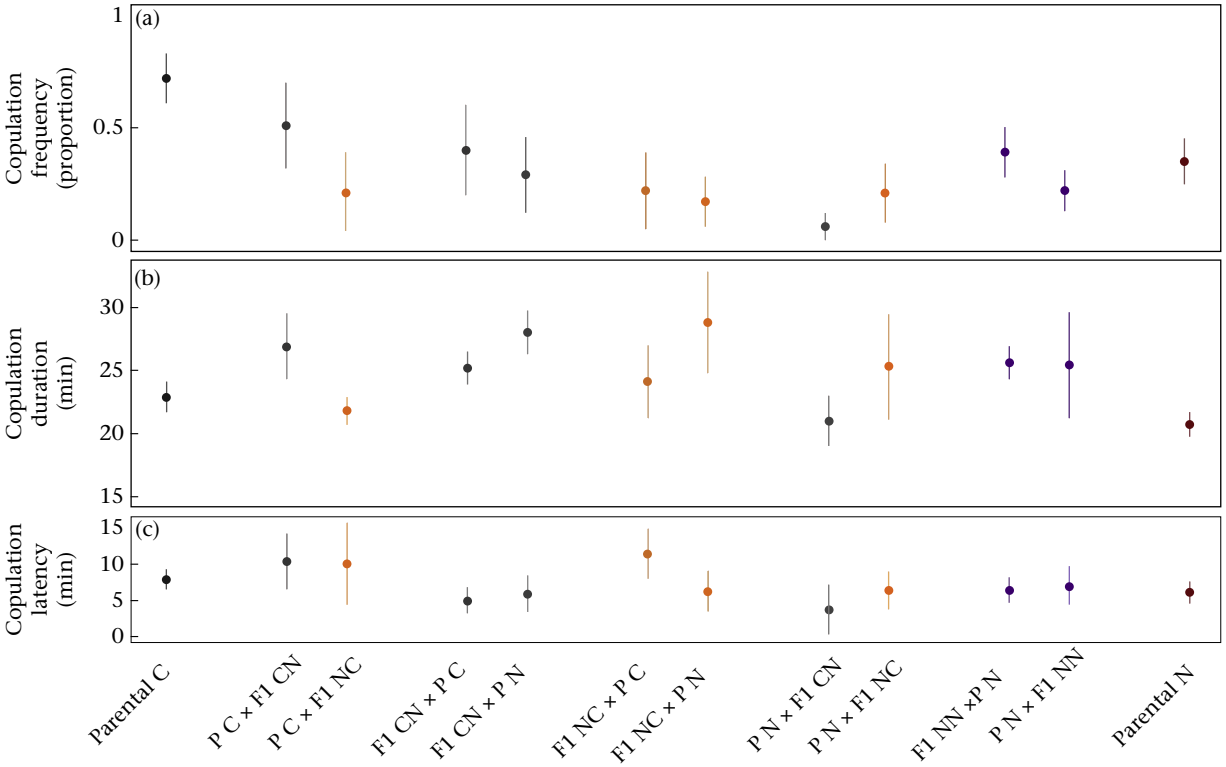

Figure A2. Comparison of copulation traits of parental and backcross pairings (mean ± 95% confidence interval). (a) Copulation frequency, (b) copulation duration and (c) copulation latency. Backcrosses with C × N hybrids are in grey, those with N × C hybrids in orange. Continental *S. neocynipsea* (N × N) backcrosses are in violet.

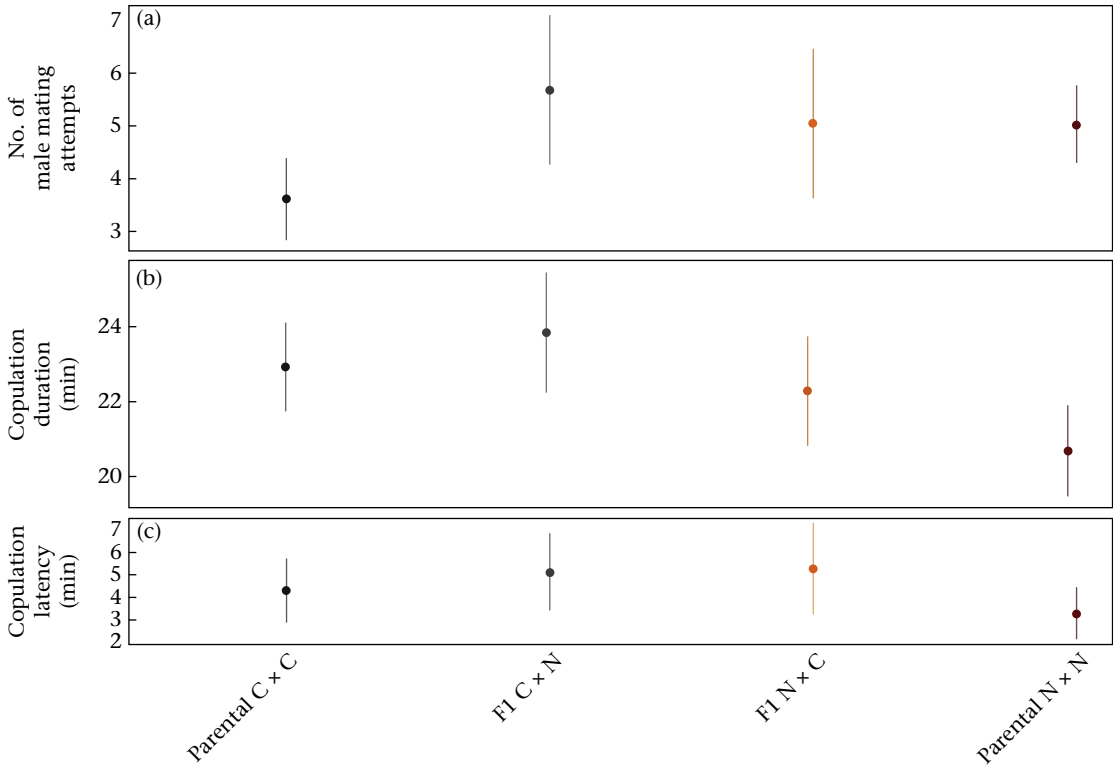

Figure A3. (a) Number of male mating attempts, (b) copulation duration and (c) copulation latency compared between conspecific *S. cynipsea* (black), conspecific *S neocynipsea* (red) and F_1 hybrid offspring (grey for C × N, orange for N × C) pairings in evaluating the inheritance pattern of behavioural traits (mean ± 95% confidence interval).